

Inverse relationship between autophagy and CTSK is related to bovine embryo quality

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Abstract

Improving the quality and the developmental competence of *in vitro* produced (IVP) embryos is an indispensable goal for assisted reproductive technology. Autophagy is a major protective mechanism for intracellular degradation of unnecessary cytoplasmic components. Autophagy ends by the fusion between autophagic vacuoles and lysosomes, allowing the degradation of the cargo by lysosomal enzymes, especially the cathepsins (CTSs). However, it is still unclear how autophagy and cathepsin K (CTSK) relate to embryo development. This study evaluated (1.) the activities of autophagy and CTSK in relation to bovine embryo quality and (2.) the effect of autophagy induction and/or CTSK inhibition on preimplantation embryo development and quality. We show here that good-quality embryos exhibited a greater autophagic activity and less CTSK activity compared to poor-quality embryos. Blastomeres of an individual embryo may vary in their quality. Good quality blastomeres showed an increased autophagic activity and decreased CTSK activity compared to poor-quality blastomeres within the same embryo at different developmental stages. Importantly, induction of autophagy and/or inhibition of CTSK improved the developmental rate (increased blastocyst and hatching rates) and the quality (increased total cell number and decreased the percentage of apoptotic cells) of IVP bovine embryos. These results demonstrate a promising approach to selectively isolate good-quality embryos and improve the efficiency of IVEP of cattle embryos.

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Introduction

In vitro embryo production (IVEP) is an efficient approach to produce and disseminate animals of superior genetic properties. Although IVEP technology has been greatly progressed in the recent years, the overall success remains lower than that in *in vivo* counterparts (Arias-Alvarez *et al.* 2011, Han *et al.* 2017). Because reduced embryo quality is the major obstacle that compromises the success of IVEP and because morphological selection of embryos is less accurate and accompanied by poor outcomes (Deniz *et al.* 2018), it is essential to improve the efficiency of *in vitro* culture (IVC) conditions and identify biomarkers that allow evaluation and selection of high-quality embryos.

Autophagy is a major pathway for intracellular degradation of cytoplasmic components (Eskelinen 2005, Mizushima 2007, Agnello *et al.* 2015). The

process starts with formation of an autophagosome that engulfs intra-cellular organelles. During the formation of autophagosomal membrane, cytoplasmic light chain 3B (LC3-I) binds to phosphatidylethanolamine to form the lipid modified conjugate (LC3-II). LC3-II is critical for autophagosome membrane expansion and fusion with the lysosome prior to the final degradation of the cargo by lysosomal enzymes (Mizushima 2007, Axe *et al.* 2008, Simonsen & Tooze 2009, Weidberg *et al.* 2011). During stress, autophagy plays a pivotal role in maintaining cellular metabolism and homeostasis by selectively degrading unnecessary cytoplasmic components to provide nutrients and energy (Wu *et al.* 2012, Singh & O'Reilly 2019). Autophagy is highly induced soon after fertilization and plays an important role during early embryonic development (Tsukamoto *et al.* 2008). Inhibition of autophagy during IVC impaired the developmental competence

of preimplantation mouse embryos (Tsukamoto *et al.* 2008, Pan *et al.* 2020) and its induction improved the viability of cloned mouse embryos (Shen *et al.* 2015). Moreover, induction of autophagy using rapamycin during IVM or IVC improved the developmental competence of parthenogenetically produced porcine embryos (Lee *et al.* 2015, Elahi *et al.* 2017) as well as promoted the preattachment development of bovine embryos (Song *et al.* 2012), indicating that regulating autophagic activity is a promising approach to improve the efficiency of IVEP. Whether autophagic activity directly correlates with the developmental capacity of bovine embryos is still unknown.

The final step of autophagy-mediated intracellular degradation is the fusion between autophagosomes and lysosomes to form autolysosomes, necessary to degrade the cargo by lysosomal enzymes, especially the cathepsins (CTs) (Galluzzi *et al.* 2014, Liu *et al.* 2017). CTs constitute a major family of lysosomal proteases which play a key role in intracellular protein turnover. (Fonovic & Turk 2014). Increased CTs activity induces lysosomal membrane damage, and their leakage into the cytoplasm results in initiating caspase3/apoptotic cascade in different cell types pathway (Krippner *et al.* 1996, Adachi *et al.* 1997, Bossy-Wetzell *et al.* 1998, Scaffidi *et al.* 1998, Vancompernelle *et al.* 1998, Balboula *et al.* 2013). We previously demonstrated that active cathepsin B (CTSB) is expressed in bovine cumulus cells, oocytes and embryos and that its activity is inversely correlated with the quality of bovine oocytes and embryos (Balboula *et al.* 2010a,b, 2013). Importantly, we and others showed that inhibition of CTSB activity during *in vitro* maturation (IVM) and IVC improved the developmental competence and quality of bovine oocytes and embryos, respectively (Bettegowda *et al.* 2008, Balboula *et al.* 2010a,b, Min *et al.* 2014, Aboelenain *et al.* 2017). Furthermore, inhibition of CTSB activity rescued the adverse effect of heat-shock during IVM, *in vitro* fertilization (IVF) or IVC on subsequent bovine embryo development (Balboula *et al.* 2013, Yamanaka *et al.* 2018). The compromising effect of CTSB on embryonic quality has also been confirmed in other species. For example, inhibition of CTSB improved the quality and developmental competence of porcine and ovine oocytes and embryos (Kim *et al.* 2015, Pezhman *et al.* 2017, Liang *et al.* 2018). Interestingly, transcript abundances of *Ctsb* and *Ctsk* were upregulated in cumulus cells surrounding low developmentally competent bovine oocytes when compared to those surrounding high developmentally competent ones (Bettegowda *et al.* 2008). CTSK (also known as cathepsin O2) is predominantly expressed in osteoclast cells and ovaries (Bromme & Okamoto 1995, Vizovisek *et al.* 2019) and its inhibition is used to treat osteoporosis in humans (Boggild *et al.* 2015). Although the involvement of CTSB in regulating oocyte and embryo quality has been established, data are lacking

about the role of CTSK in regulating the developmental competence of preimplantation mammalian embryos and whether it correlates with embryonic quality.

Here, we find that CTSK and autophagic activities exhibit opposite patterns during early embryonic development. CTSK activity was increased in poor-quality embryos, whereas autophagic activity was decreased in good-quality ones. Embryonic blastomeres vary in their quality. Inside the same embryo, we found that poor-quality cells showed high CTSK activity, while good-quality cells showed high autophagic activity. We then asked whether inhibition of CTSK activity and/or autophagy induction improves embryo developmental competence and quality. Supplementation of the IVC medium with rapamycin (Rap), an autophagy inducer, and/or odanactib (OD; a cell permeable CTSK inhibitor) improved the developmental competence and quality of bovine embryos. This study is the first to demonstrate the inverse relationship between CTSK and autophagic activities in relation to embryo quality and to provide evidence that manipulating CTSK/autophagy ratio is a promising approach to improve the developmental competence and quality of preimplantation mammalian embryos.

Materials and methods

Unless otherwise specified, all the chemicals and reagents used in this study were purchased from the MilliporeSigma, MO, USA.

Oocytes collection and *in vitro* maturation (IVM)

Holstein cow ovaries were collected from a local abattoir (Hayakita branch, Abira, Hokkaido). Aspiration of cumulus oocyte complexes (COCs) from medium-sized follicles (2–8 mm) was performed using an 18-gauge needle followed by washing three times in the maturation medium, Tissue Culture Medium (TCM)-199 (Invitrogen) supplemented with 5% (V/V) fetal calf serum and follicle stimulating hormone (0.02 units/mL). COCs were then matured in a TCM-199 (50 COCs/500- μ L drop) under mineral oil for 22 h at 38.5°C in a humidified atmosphere of 5% CO₂ in air.

In vitro fertilization (IVF) and *in vitro* Culture (IVC)

Straws of Holstein bull semen (from two fertile bulls) were thawed in a water bath at 37°C for 30 s. Separation and washing of the actively motile sperm were done by centrifugation at 1800 g for 7 min in Brackett and Oliphant (BO) medium (Brackett & Oliphant 1975) containing 2.5 mM theophylline (Wako Pure Chemical Industries) and 7.5 μ g/mL heparin sodium salt (Nacalai Tesque, Inv., Kyoto, Japan). After centrifugation, the sperm pellet was diluted with BO medium to get a final concentration of 5×10^6 spermatozoa per mL. COCs were then incubated and fertilized by active motile sperm in droplets of modified BO's isotonic medium (10 COCs/100 μ L) containing 6 mg/mL fatty acid-free BSA and

2.5 mM theophylline for 18 h at 38.5°C under a humidified atmosphere of 5% CO₂ in air. After fertilization, putative zygotes were denuded mechanically by repeated pipetting (Aono *et al.* 2013) followed by culturing in 50 µL drops (20 putative zygotes/drop) of modified synthetic oviduct fluid medium supplemented with amino acid solution, 10 µM insulin, 1 mM glucose, and 3 mg/mL fatty acid-free BSA at 38.5°C under 5% CO₂, 5% O₂ and 90% N₂.

Detection of intracellular CTSK activity

Intracellular CTSK activity was detected by using a Magic red CTSK assay kit (Immunochemistry Technologies LLC, Bloomington, MN, USA) following the manufacturer instructions with some modifications. Briefly, embryos were incubated with the reaction mix (2 µL of DMSO-diluted stock solution in 500 µL culture medium) for 25 min in a humidified atmosphere of 5% CO₂ at 38.5°C. Nuclei staining was carried out by adding Hoechst 33342 (25 µg/mL) to the same staining solution for an additional 5 min. Embryos were then rinsed in PBS containing 0.5 mg/mL polyvinylpyrrolidone (PVP-PBS; Nacalai Tesque) prior to mounting onto a glass slide and observed under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Nuclei were observed following excitation at 375 nm and intracellular CTSK activity was detected at 585 nm excitation. Images of CTSK activity within the same experiment were captured under the same parameters. Images were analyzed and the intensity of fluorescence was quantified using image J software (National Institutes of Health, Bethesda, MD, USA), keeping the processing parameters identical in all groups.

Detection of autophagy activity

Autophagic activity was detected using Cyto-ID Autophagy Detection kit (Enzo Life Sciences, Farmingdale, NY, USA) following the manufacturer's instructions with some modifications. Briefly, embryos were incubated with the reaction mix (1 µL in 500 µL culture medium) at 38.5°C for 25 min. DNA was stained by adding Hoechst 33342 (25 µg/mL) to the same staining solution for an additional 5 min. Embryos were then rinsed in PVP-PBS, mounted onto a glass slide, and imaged by fluorescence microscope (Leica) using 375 nm and 495 nm excitation filters to observe DNA and autophagy activity, respectively. The images were analyzed using the Image J software.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)

Day 7 blastocysts were assessed for apoptosis using TUNEL assay kit (*in situ* cell death detection kit; Roche Applied Science). Blastocysts were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 40 min, rinsed three times in PVP-PBS, and then permeabilized in PVP-PBS containing 0.5% TritonX-100 for 20 min. After washing three times in PVP-PBS for 5 min each, fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 38.5°C. After incubation, blastocysts were washed three times in PVP-PBS

for 5 min each, followed by mounting onto glass slides using mounting solution containing DAPI (Vectashield with DAPI; Vector Laboratories, Burlingame, CA, USA). Pretreatment of blastocysts with DNase I recombinant served as a positive control, while omitting fluorescein-dUTP served as a negative control. The fluorescence of the fragmented DNA ends was detected by a fluorescence microscope (Leica) using 495 nm excitation filter. The percentage of apoptotic positive cells was calculated for each embryo as follows: number of TUNEL-positive nuclei/total number of nuclei in blastocyst ×100.

Statistical analysis

One-way ANOVA and Student *t*-test were used to evaluate the differences between groups using GraphPad Prism. One-way ANOVA was used to compare cleavage, blastocyst, and hatching rates among treated groups. ANOVA test was followed by the Tukey post hoc test. All percentage data were arcsine transformed prior to statistical analysis. The differences of *P* < 0.05 were considered significant. Each experiment was replicated at least three times. The data were expressed as means ± S.E.M.

Experimental design

Evaluation of CTSK and autophagic activities in relation to the developmental competence and quality of bovine embryos

After IVF, bovine embryos were cultured for 5 days. Day 2 and 5 embryos were divided into two groups ('good' and 'poor' quality embryos) according to their developmental kinetics and morphology. In brief, embryos with normal morphology (symmetrical without any fragmentation) and fast development (have the developmental stage corresponding to each time point) (Lindner & Wright 1983) were classified as good-quality embryos. On the other hand, slow-developing embryos that are lagging one cell cycle behind each time point with or without poor morphology (asymmetry and fragmentation) were classified as poor-quality embryos (Dinnyes *et al.* 1999, Lonergan *et al.* 1999, Yoshioka *et al.* 2000, Lequarre *et al.* 2003, Vandaele *et al.* 2007). To evaluate whether CTSK and autophagic activities are related to the quality of bovine embryos, good and poor-quality embryos were co-stained with Magic red CTSK assay kit and/or Cyto-ID Autophagy detection kit. Stained embryos were imaged by a fluorescence microscope at 495 nm and 585 nm excitation to observe autophagy and CTSK activity, respectively.

Effect of autophagy induction and/or CTSK inhibition on the developmental competence and the quality of bovine preimplantation embryos

To evaluate the effect of autophagy induction on the developmental competence of preimplantation embryos, putative zygotes (day 0) were cultured with a natural inducer of autophagic activity (Rap) at different concentrations (10, 50, or 100 nM). To evaluate the effect of CTSK inhibition, different concentrations (1, 5, and 10 µM) of OD (MK-0822,

Selleckchem, USA) were used. Both drugs (Rap or OD) were dissolved in DMSO and added to the IVC medium at day 0 for 7 days. DMSO-treated oocytes were employed as controls. The efficiency of Rap to stimulate autophagic activity and OD to inhibit CTSK was assessed in day 1 embryos using Cyto-ID Autophagy detection kit and magic red CTSK assay kit, respectively. The developmental competence was assessed by evaluating cleavage (day 2), blastocyst (day 7; including early and expanded), and hatching rates (day 7). The concentration that achieves the highest developmental rate of each treatment was selected for an additional experiment where the effect of combined autophagy induction and CTSK inhibition on developmental competence was evaluated. The quality for the produced blastocyst was assessed by evaluating the total cell number and the percentage of apoptotic cells in day 7 blastocysts.

Results

The quality of bovine embryo is associated with an inverse relationship between autophagy and CTSK activity

To investigate the relationship between embryo quality and both CTSK and autophagic activities, days 2 or 5 embryos were classified into good and poor-quality embryos according to the criteria described previously prior to evaluating the activities of CTSK and autophagy using Magic-red CTSK Kit and Cyto-ID, respectively. We confirmed the specificity of both reagents in our system, where inhibiting CTSK using a cell permeable CTSK inhibitor (OD) reduced CTSK activity (Supplementary Fig. 1A and B, see section on [supplementary materials](#) given at the end of this article) and autophagy induction using Rap increased Cyto-ID autophagy signals (Supplementary Fig. 1C and D). Importantly, at each time point, autophagy exhibited an opposite pattern, where autophagy activity was greater in good-quality embryos when compared to poor-quality embryos (Fig. 1A, B, E and F). On the other hand, CTSK activity was greater in poor-quality embryos when compared to good-quality ones (Fig. 1A, C, E and G). Accordingly, autophagy/CTSK ratio was greater in good-quality embryos than poor-quality embryos (Fig. 1D and H).

Inverse relationship between autophagy and CTSK activity is represented within blastomeres of the same embryo

After fertilization, the 1-cell embryo divides mitotically. The produced cells from this cleavage are called blastomeres. During the previous experiment, we noticed that some embryos show a heterogeneity of autophagy and CTSK activity among blastomeres. The quality of embryonic blastomeres differ within the same embryo. To investigate whether the inverse relationship between CTSK and autophagic activities is related to blastomere quality, bovine embryos at

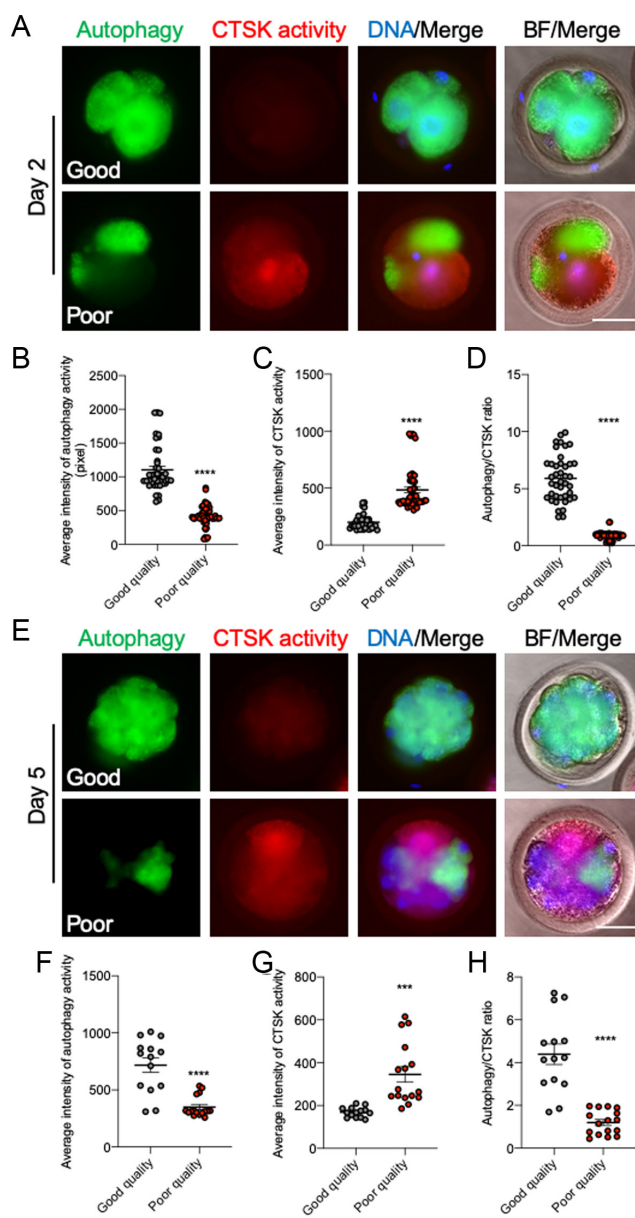


Figure 1 The quality of bovine embryos associates with inverse relationship between autophagy and CTSK activity. Day 2 and 5 bovine embryos were divided according to their quality into good and poor-quality embryos. Bovine embryos were co-stained with Cyto-ID Autophagy detection kit and Magic red CTSK assay kit to detect autophagy activity (green) and CTSK activity (red), respectively. DNA was detected via Hoechst staining (blue). Bright field (BF) images are shown in merge. Shown are representative images (scale bar, 100 μm) for good (upper row) and poor-quality (lower row) embryos at day 2 (A) and day 5 (E) after IVF. Average intensity of autophagy activity, CTSK activity as well as autophagy/CTSK ratio were quantified in day 2 (B, C and D, respectively) and day 5 embryos (F, G and H, respectively). Experiments were repeated at least three times and total number of analyzed embryos is 154. The data are expressed as mean ± s.e.m. and Student *t*-test was used to analyze the data. Values with asterisks vary significantly, ****P*<0.001, *****P*<0.0001.

different stages (2-cell, 4 to 8-cell, and morula) of embryonic development were co-stained with Cyto-ID and Magic-red CTSK Kit to assess autophagy and CTSK activity, respectively. Interestingly, within each developmental stage, we found that morphologically normal blastomeres (light homogenous cytoplasm, regular plasma membrane borders, and without signs of vesiculation or fragmentation) (Hardarson *et al.* 2001) had a high level of autophagy and a low level of CTSK activity when compared to morphologically abnormal blastomeres in 2-cell (Fig. 2A), 4 to 8 cell (Fig. 2B), and morula (Fig. 2C) embryos.

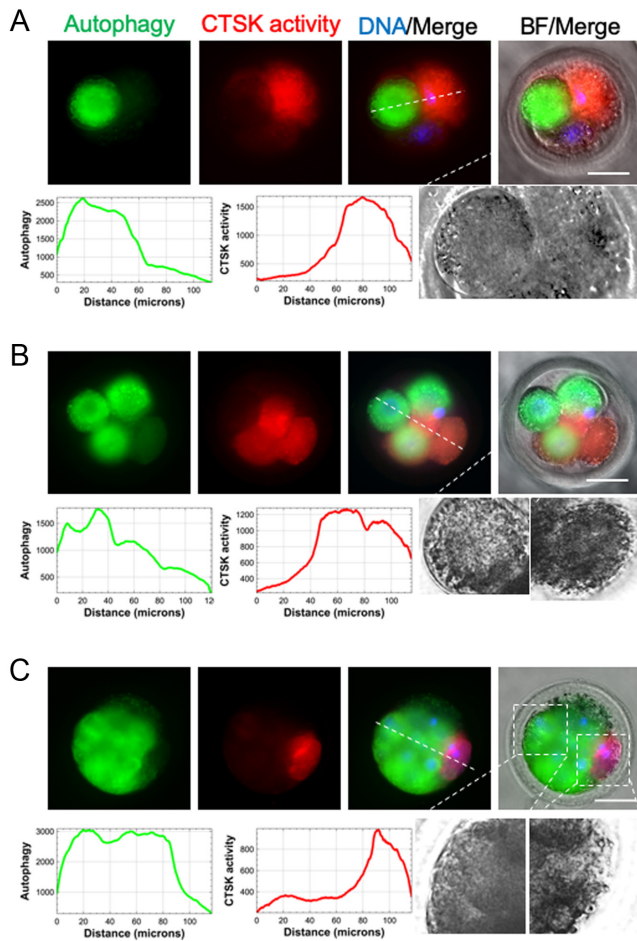


Figure 2 The quality of blastomeres of the individual embryo follows an inverse relationship between autophagy and CTSK activity. Bovine embryos at different stages (2-cell (A), 4–8-cell (B), and morula (C)) of embryonic development were co-stained with Cyto-ID Autophagy detection kit and Magic red CTSK assay kit to detect autophagy activity (green) and CTSK activity (red), respectively, in individual blastomeres. DNA was detected via Hoechst staining (blue). Bright field (BF) images are shown in merge. Poor-quality blastomeres (irregular and vesiculated) showed higher CTSK activity and lower autophagy than good-quality blastomeres. Lower panels: Examples of quantitative assessment of autophagy (green) and CTSK activities (red) using the ‘plot profiles’ function in ImageJ. Shown are representative images (scale bar, 100 μm). Experiments were repeated at least three times and total number of analyzed embryos is 154.

Inhibition of CTSK activity and/or induction of autophagy activity during IVC improves subsequent embryonic development

Given the inverse relationship between CTSK activity and embryo quality, we hypothesized that the inhibition of CTSK during IVC might promote the subsequent embryonic development. To this end, different concentrations of a highly selective CTSK inhibitor, OD (Hao *et al.* 2015a,b, Bahuguna *et al.* 2016, Li *et al.* 2019), was added to the IVC medium and the developmental rates of bovine embryos were examined. Treatment of bovine embryos with OD during IVC did not affect the cleavage rate (Fig. 3A). However, the embryos cultured in the presence of 5 μM OD displayed an increase in the rate of embryonic development to the blastocyst stage (Fig. 3B). Importantly, hatching rates were increased when embryos were cultured in a medium supplemented with 5 μM OD (Fig. 3C).

Culturing bovine embryos with Rap during IVC had no effect on the cleavage rate (Fig. 4A). However, the embryos cultured in the presence of 50 or 100 nM Rap showed an increase in the blastocyst rate (Fig. 4B). Hatching rates were increased when embryos were

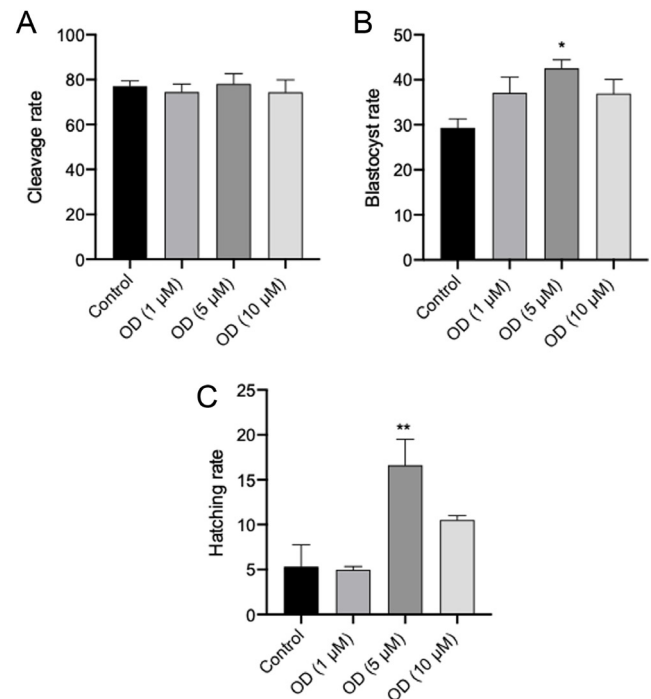


Figure 3 Effect of odanactib (OD) treatment during *in vitro* culture on subsequent cleavage, blastocyst, and hatching rates. After *in vitro* fertilization, putative zygotes were cultured for 7 days in culture medium supplemented without (DMSO control) or with 1, 5, or 10 μM OD (CTSK inhibitor). Cleavage rate (A) was assessed on day 2, whereas blastocyst (B) and hatching rates (C) were observed on day 7. Experiments were repeated four times and total number of embryos is 333. The data are expressed as mean ± S.E.M. and values with asterisks vary significantly, **P* < 0.05, ***P* < 0.01.

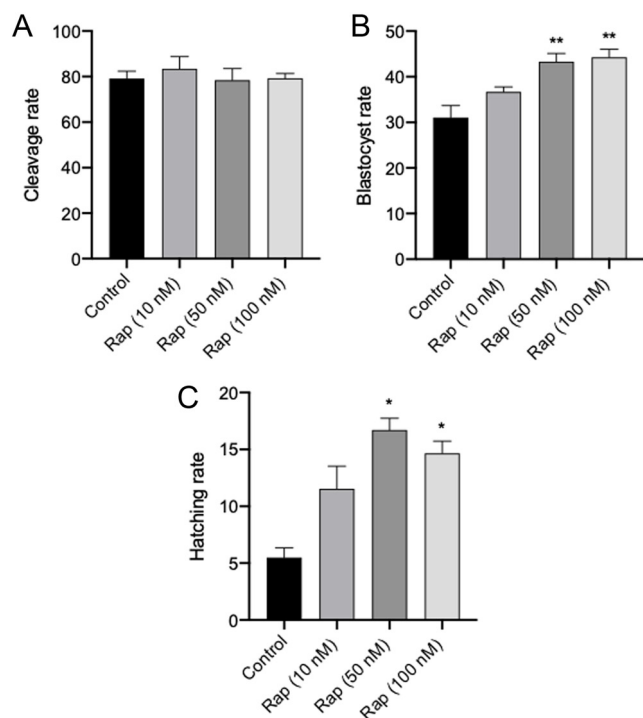


Figure 4 Effect of rapamycin (Rap) treatment during *in vitro* culture on subsequent cleavage, blastocyst, and hatching rates. After *in vitro* fertilization, putative zygotes were cultured for 7 days in culture medium supplemented without (DMSO control) or with 10, 50, or 100 nM Rap (autophagy inducer). Cleavage rate (A) was assessed on day 2, whereas blastocyst (B) and hatching rates (C) were observed on day 7. Experiments were repeated three times and total number of analyzed embryos is 274. The data are expressed as mean \pm S.E.M. and values with asterisks vary significantly, * $P < 0.05$, ** $P < 0.01$.

cultured in a medium supplemented with 50 or 100 nM Rap (Fig. 4C).

Because CTSK activity is inversely correlated with autophagic activity and because inhibition of CTSK activity or induction of autophagy improved the developmental rates of bovine embryos, we hypothesized that inhibition of CTSK and induction of autophagy simultaneously might have an additional improving effect. Strikingly, supplementation of the IVC medium with both 5 μ M OD and 50 nM Rap (the lowest concentrations that achieved the highest developmental rates) increased the blastocyst and the hatching rates ($P < 0.0001$, Fig. 5B and C) but not the cleavage rates (Fig. 5A) when compared to control, OD, or Rap alone.

Average total cell number and the number of TUNEL apoptotic cells are considered valuable markers to judge embryo quality (Neuber *et al.* 2002, Fouladi-Nashta *et al.* 2005). To investigate whether OD and/or Rap improves embryo quality, total cell number and the apoptotic status of day 7 blastocysts were assessed. The total cell number of blastocysts 7 days after addition of OD and/or Rap was increased than that in the control ones (Fig. 6A and B). The percentage of apoptotic cells

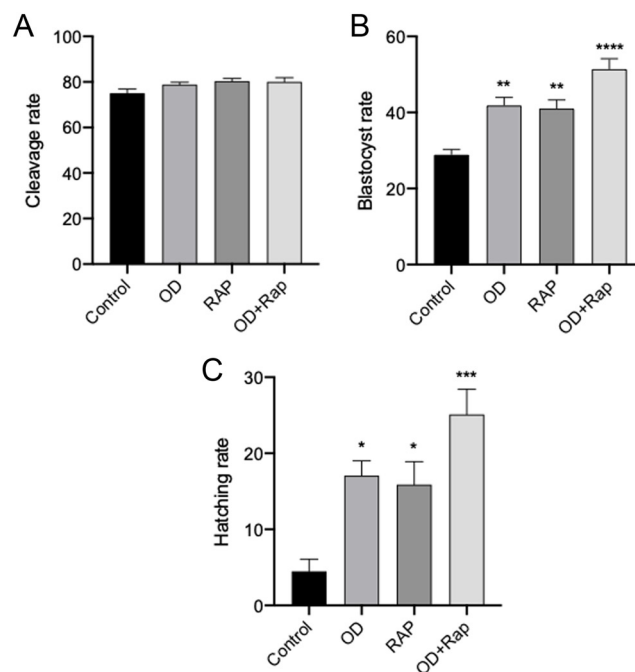


Figure 5 Rapamycin (Rap) and/or odanactib (OD) treatment during *in vitro* culture improved blastocyst and hatching rates of bovine embryos. After *in vitro* fertilization, putative zygotes were cultured for 7 days in culture medium supplemented with DMSO (control), OD (5 μ M), Rap (50 μ M), or OD + Rap. Cleavage rate (A) was assessed on day 2, whereas blastocyst (B) and hatching rates (C) were observed on day 7. Experiments were repeated five times and total number of analyzed embryos is 457. The data are expressed as mean \pm S.E.M. and values with asterisks vary significantly, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

(TUNEL-positive cells) in the blastocysts derived from embryos cultured with OD and/or Rap was less than that in the control group (Fig. 6A and C). Taken together, these results strongly indicate that manipulating CTSK and autophagy levels is a promising approach to improve the developmental capacity and quality of IVP bovine embryos.

Discussion

In vitro production of embryos is an effective technology to improve genetic properties of animals and their production. However, major limitations of IVEP success are reduced developmental rate and reduced quality of the produced embryos. In this report, we found that CTSK activity is increased in poor-quality embryos when compared to good-quality ones. On the other hand, autophagy was increased in good-quality embryos than poor-quality embryos. Importantly, inhibition of CTSK activity and/or induction of autophagy during IVC improved the developmental competence and the quality of the produced embryos.

Autophagy is a process where cells degrade their unnecessary cellular components to provide energy and

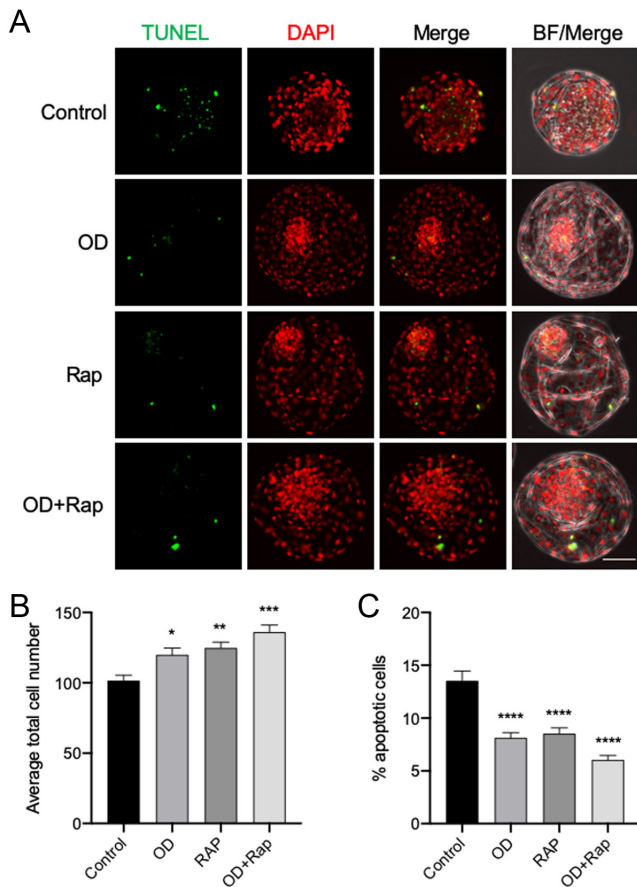


Figure 6 Effect of rapamycin (Rap) and/or odanactib (OD) treatment during *in vitro* culture on total cell number and apoptotic status of bovine blastocysts. After *in vitro* fertilization, putative zygotes were cultured for 7 days in culture medium supplemented with DMSO (control), OD (5 μ M), Rap (50 nM), or OD+Rap. (A) TUNEL staining (green) was used to detect apoptotic cells, while DAPI staining (red, pseudo color) was used for DNA detection and total cell number assessment. Shown are representative images (scale bar, 100 μ m). (B) Quantification of average total cell number. (C) Quantification of TUNEL apoptotic positive cells. Experiments were replicated three times and total number of blastocysts examined is 84. The data are expressed as mean \pm s.e.m. and values with asterisks vary significantly, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

nutrients (Eskelinen 2005, Mizushima 2007, Agnello *et al.* 2015). Autophagy is upregulated after fertilization in early mouse embryos (Tsukamoto *et al.* 2008). The relative expression of autophagy marker, LC3, was abundant in the zygote, reached the peak at the 2-cell and 4-cell stages, and gradually decreased from the morula to the blastocyst stage (Tsukamoto *et al.* 2008). We found a positive association between autophagy activity and bovine embryo quality, suggesting that autophagy activity is essential for early embryonic development. Indeed, the fertilization of mouse oocytes lacking Autophagy Related 5 (ATG5) with Atg5^{-/-} sperm resulted in a failure of embryonic development beyond 4-cell stage (Tsukamoto *et al.* 2008). Moreover, induction of autophagy using rapamycin and pp242

during somatic cell nuclear transfer (SCNT) enhanced the development of SCNT embryos to the blastocyst stage (Shen *et al.* 2015). In bovine embryos, autophagy is inversely correlated with endoplasmic reticulum stress (Song *et al.* 2012). This finding comes in consistency with our observations of high autophagy activity in good-quality embryos because early bovine embryo development requires appropriate balance between autophagy and endoplasmic reticulum stress (Song *et al.* 2012). The improving effect of autophagy induction using Rap during IVC further confirms the importance of autophagy during early development, likely through degrading unrequired maternal RNAs and proteins after maternal zygotic activation as well as providing nutrients and energy (Mizushima *et al.* 2004, Tsukamoto *et al.* 2008, Mizushima & Levine 2010).

Although lysosomal cathepsins are ubiquitously expressed in different cell types, CTSK (cathepsin O2) showed predominant levels of expression specifically in osteoclast cells, cancer cells, and ovaries (Bromme & Okamoto 1995, Vizovisek *et al.* 2019). The role of CTSK in the ovary has not been studied. Here, we showed that CTSK activity is expressed in early stage bovine embryos, albeit increased in poor-quality ones. Our previous studies demonstrated that CTSB activity, another member of lysosomal cysteine protease family, is inversely correlated with the quality of bovine embryos (Balboula *et al.* 2010b). Exposure of lysosomes to a moderate stress causes CTSB leakage into the cytoplasm (Brunk *et al.* 1997). However, increased intra-lysosomal CTSB activity can promote lysosomal membrane permeability leading ultimately to its leakage (Werneburg *et al.* 2002, Johansson *et al.* 2010). In both cases, leakage of CTSB into the cytoplasm is the key player for initiating caspase 3/apoptotic pathway (Krippner *et al.* 1996, Adachi *et al.* 1997, Bossy-Wetzel *et al.* 1998, Scaffidi *et al.* 1998, Vancompernelle *et al.* 1998). Similar to our CTSK results, inhibition of CTSB activity during IVC decreased the number of TUNEL-positive (apoptotic) cells and improved the developmental competence and quality of bovine embryos (Balboula *et al.* 2010b). Taken together, CTSK seems to have the same compromising effect as CTSB does on the quality of bovine embryos. OD selectively binds to and inhibits CTSK activity in different cell types (Hao *et al.* 2015a,b, Bahuguna *et al.* 2016, Li *et al.* 2019). To our knowledge, OD does not have the ability to inhibit other cathepsins or cysteine proteases (Gauthier *et al.* 2008). Owing to its ability to inhibit CTSK and its ability to reduce bone resorption while maintaining bone formation, OD was used to treat osteoporosis in humans (Boggild *et al.* 2015). Here, we found that CTSK inhibition by OD during IVC improved preimplantation embryonic development in bovine, supporting the notion that regulating lysosomal CTSs is a promising approach to improve the efficiency of IVEP.

Identifying markers for good-quality embryos has been an essential goal for efficient IVEP either in animals or

humans. We found an opposite pattern between CTSK and autophagy in relation to embryo quality. Good-quality but not poor-quality embryos exhibited high autophagy and low CTSK activity. This result suggests that high CTSK activity could be used as a marker of poor-quality embryos, whereas high autophagy activity indicates good-quality embryos. Although each of CTSK and autophagy activities could be used a quality marker, the ratio of autophagy/CTSK within the same developmental stage is a more accurate predictor of embryo quality, as it excludes the possible effect of fluorescence bleaching and imaging variations among different experiments that require live cells. Determining the cut-off point of this ratio will be a helpful tool, as a quality marker to improve the efficiency of assisted reproductive technology.

The relationship between apoptosis and autophagy is still controversial. Although autophagy inhibits apoptosis in certain type of cells, it can also stimulate apoptosis in special cases (Marino *et al.* 2014). Although we have not addressed the underlying mechanism of this opposite pattern between lysosomal CTSK and autophagy, different possibilities could be considered. One possibility is that the activity of autophagy or CTSK negatively regulates the other. For example, suppression of lysosomal enzymes induces autophagy via a feedback down-regulation of MTOR Complex 1 (Li *et al.* 2013). On the other hand, autophagy has the ability to inhibit apoptosis by blocking the activation of cytoplasmic caspases (Thorburn 2008, Song *et al.* 2017). However, the additive improving effect of combined OD+Rap over OD or Rap alone suggests that autophagy and CTSK have non-overlapping pathways to determine the quality of early stage embryos. Further investigations are required to dissect these possibilities. In conclusion, the results reported here reveal that autophagy and CTSK activities could be used as markers of bovine embryo quality. Moreover, induction of autophagy and/or inhibition of CTSK during IVC are promising approaches to improve the developmental rate and quality of IVP bovine embryos.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0036>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A Z B and M T conceived the study. A Z B performed the experiments. A Z B, M A, J L, M A, H B, and M K analyzed data and prepared the figures. A Z B and M T wrote the paper.

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